Appendix A

A Bispecific Monoclonal Antibody Directed Against Both the Membrane-Bound Complement Regulator CD55 and the Renal Tumor-Associated Antigen G250 Enhances C3 Deposition and Tumor Cell Lysis by Complement[1]

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Tumor cells may inhibit the induction of a complement-mediated inflammatory response through overexpression of membrane-bound regulators of complement activation. Therefore, it is of interest to determine the most efficient approach to block these membrane-bound complement regulators on tumor cells. In the present study, we first generated a bispecific mAb directed against both CD55, using the functional blocking mAb MBC1, and the highly expressed HLA class I molecule as a model for a tumor-associated Ag, using the mAb W6/32. Tumor cells opsonized with bispecific mAb W6/32*MBC1, then exposed to complement and subsequently stained for C3 deposition, were assessed by flow cytometric analysis. We found that opsonization with W6/32*MBC1 resulted in a 92% enhancement of C3 deposition on renal tumor cells as compared with opsonization with W6/32 alone and a 17% enhancement of the C3 deposition as compared with incubation with a mixture of both parental mAb. Based on these results, we developed a bispecific mAb recognizing both CD55 and the relatively low expressed renal tumor-associated Ag G250. Increasing concentrations of the bispecific mAb G250*MBC1 resulted in a 25 to 400% increase in C3 deposition on renal tumor cells as compared with C3 deposition in the presence of the parental mAb G250 alone. G250*MBC1 enhanced C3 deposition by 21% in comparison with a mixture of both parentals. Furthermore, opsonization of tumor cells with G250*MBC1 rendered these cells more sensitive to complement-mediated lysis. In conclusion, the bispecific mAb G250*MBC1 induces deposition of C3 and tumor cell lysis more efficiently than G250 alone. *The Journal of Immunology,* **1998, 160: 3437–3443.**

Renal cell carcinomas (RCC)[3] are chemo- and radiotherapy resistant (1). The occurrence of spontaneous regressions suggests the involvement of the immune system in tumor eradication. Indeed, RCC partially respond to immunotherapeutic treatment. In addition to the widely applied IL-2 based immunotherapy of RCC (2–4), some trials have been undertaken using the mAb G250 in the treatment of RCC (5, 6). MAb G250 recognizes the G250 Ag, which is expressed on ~90% of primary RCC and 82% of metastatic RCC lesions but not on normal kidney cells. Ab localization studies have shown that mAb G250 localizes specifically to G250 Ag-positive RCC and that uptake in the tumors is very high. Cross-reactivity of mAb G250 with normal tissues is restricted to gastric mucosal cells and cells of the larger bile ducts (7). So far, no major improvements have been seen in patients after treatment with ^{131}I-labeled G250 (phase I/II trial), although some patients with disseminated inoperable disease remained stable (5).

The low response rate of tumors found thus far following immunotherapeutic treatment using complement-activating mAb against tumor-associated Ags (8) may be attributable to overexpression of membrane-bound regulators of complement activation (mRCA) by tumor cells. These mRCA are expressed on normal host cells to protect them from uncontrolled complement-mediated injury (9). The fact that tumor cells have been shown to overexpress mRCA (10–14) suggests a mechanism by which tumors can protect themselves against the effects of complement activation.

Blocking mRCA on tumor cells could improve the efficacy of immunotherapy with complement-activating mAb. In vivo, the role for complement in eradication of a tumor could be more to elicit an immune response at the tumor site by which effector cells are attracted than direct cytolysis of the tumor cells. Based on this assumption, bispecific mAb were produced with one arm directed against a tumor-associated Ag and the other arm against the mRCA, CD55. This complement regulatory protein is involved in the irreversible dissociation of C3/C5 convertases (15). Blocking the function of CD55 results in membrane deposition of C3b and generation of chemotactic factors such as C5a.

In this study, the applicability of a bispecific mAb for future immunotherapy was evaluated. We first generated a bispecific mAb simultaneously directed against CD55 and the highly expressed HLA class I molecule as a model for a tumor-associated Ag. Based on the positive results obtained, a bispecific mAb directed against both CD55 and the renal tumor-associated Ag G250, which is expressed at a lower density than HLA class I, was generated.

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[3] Abbreviations used in this paper: RCC, renal cell carcinomas; mRCA, membrane-bound regulators of complement activation; NHS, normal human serum; BRC, baby rabbit complement; GAM, goat anti-mouse; PI, propidium iodide; PIPLC, phosphatidylinositol-phospholipase C.

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Materials and Methods

Hybridomas and mAbs

W6/32 hybridoma cells, producing anti-HLA class I mAb (IgG2a), were obtained from the American Type Culture Collection (ATCC) (Rockville, MD), MBC1 hybridoma cells, producing anti-CD55 mAb (IgG1), were kindly provided by Dr. C. L. Harris and Dr. B. P. Morgan (University of Wales, Cardiff, U.K.); and G250 hybridoma cells, producing anti-G250 mAb (IgG1 and switch variant IgG2a), were developed at our department (16). Hybridoma cells were cultured in RPMI 1640 (ICN Biomedicals, Costa Mesa, CA) supplemented with 10% heat-inactivated FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine (complete RPMI medium). GB24 (IgG1), a mAb blocking the function of CD46, was a kind gift from Drs. T. J. Oglesby and J. P. Atkinson (Washington University School of Medicine, St. Louis, MO); mAb BRIC229 (IgG2b), blocking the function of CD59, was purchased from International Blood Group Reference Laboratory (IBGRL) (Bristol, U.K.).

Complement source

Normal human serum (NHS) was obtained from a healthy AB donor. Baby rabbit complement (BRC) was purchased from Pelfreeze (Brown Deer, WI). Aliquots of sera were stored at −70°C until use.

Tumor cell lines

The human renal carcinoma cell lines SK-RC-7 and SK-RC-52 (both obtained from the Memorial Sloan Kettering Cancer Institute, NY) and the colon adenocarcinoma cell line LS180 (ATCC) were cultured in 50% DMEM (Life Technologies, Breda, the Netherlands)/50% RPMI 1640 containing 25 mM HEPES with the above mentioned supplements. The HLA class I-deficient hemopoietic cell line K562 was obtained from the ATCC. The CD55/CD59-deficient hemopoietic cell line NALM-6 was a kind gift of Dr. S. F. Vervoordeldonk (CLB, Amsterdam, The Netherlands). These cell lines were cultured in complete RPMI medium.

Generation of quadroma cells

Quadroma cells were generated as described by van Dijk et al. (17). Briefly, the MBC1 hybridoma was incubated with increasing amounts (10^{-7}-10^{-4} M) of 8-azaguanine (Life Technologies) to induce hypoxanthine-guanine phosphoribosyl transferase (HGPRT) deficiency. The W6/32 and G250 hybridomas were incubated with increasing amounts (10^{-7}-10^{-4} M) of 5-bromodeoxyuridine (Sigma Chemicals, St. Louis, MO) to induce thymidine kinase (TK) deficiency. Quadroma cells were generated by adding PEG-1500 to equal numbers (5×10^6) of HGPRT-deficient MBC1 cells and either TK-deficient W6/32 or G250 cells. The generated quadroma cells were cultured in RPMI medium, containing 0.1 mM hypoxanthine, 7.7 μM aminopterin, and 16 μM thymidine. Cell cultures were cloned in 96-well microtiter plates (Greiner, Alphen aan de Rijn, the Netherlands), each well statistically containing 0.3 cell. Quadroma clones producing bi-isotypic mAbs with functional binding sites were subcloned. Quadroma clones were selected for further study, and bi-isotypic mAb were purified from the supernatant using affinity chromatography on a protein A column. The percentage of bispecific mAb present in the bi-isotypic fraction was determined by HPLC on a Mono-S column. The bi-isotypic fractions of W6/32*MBC1 and G250*MBC1 consisted of 15 and 25% bispecific mAb, respectively.

Isotype-specific ELISA

Microtiter plates (96-well) were coated for 2 h at 37°C with rat anti-mouse IgG1 (Sanbio, Uden, the Netherlands) at a concentration of 2.5 μg/ml in 0.1 M $NaHCO_3$, pH 9.6. Serial dilutions of culture medium in PBS containing 0.05% Tween 20 (PBS/T) and 1% BSA were incubated in the coated microtiter plates for 1 h at 37°C. Bi-isotypic mAb was detected using goat anti-mouse (GAM) IgG2a conjugated with horseradish peroxidase (Southern Biotechnology Associate, Birmingham, AL). Cross-reactivity with coated rat Ig was blocked with 10% rat serum in PBS/T. The assay was developed with 3,5,3′,5′-tetramethyl-benzidine in 0.11 M sodium acetate (pH 5.5, 100 μg/ml) and freshly added H_2O_2 for 5 min at room temperature. The concentration of monospecific IgG1 heavy chain containing parental mAb was measured with a κ-chain-specific GAM conjugated with horseradish peroxidase (Sanbio). To measure the concentration of monospecific mAb, the procedure was altered by coating with GAM-IgG2a (Southern Biotechnology Associates). For determination of the mAb concentration, IgG1- and IgG2a standards, obtained from Centocor Leiden (Leiden, The Netherlands), were used.

Flow cytometry

Flow cytometric analysis was performed according to the method described by Corver et al. (18). In brief, tumor cells (2×10^5) were incubated with 100 μl mAb in PBS containing 0.5% BSA (PBS/BSA) for 30 min at 4°C. Cells were washed twice with PBS/BSA and incubated with 100 μl of a predetermined dilution of a FITC-labeled GAM-F(ab′)$_2$-IgG for 30 min at 4°C. After washing, the cells were resuspended in 250 μl PBS/BSA containing 1 μg/ml propidium iodide (PI) to stain dead cells. Samples were measured on the FACScan (Becton Dickinson, Mountain View, CA) using the software program FACScan. Ten thousand events were counted.

Phosphatidylinositol-phospholipase C treatment

LS180 cells were suspended in 50% DMEM/50% RPMI 1640 containing 1% BSA and 0.1 U/ml PIPLC (*Bacillus thurigiensis*; ICN Biomedicals, Zoetermeer, The Netherlands) and incubated for 1 h at 37°C. Cells were washed twice with 50% DMEM/50% RPMI 1640 containing 1% BSA and used for conjugate formation assays.

Conjugate formation and immunofluorescence

FITC labeling. Tumor cells (5×10^6) were incubated in 1 ml of medium containing 10 μg/ml FITC for 30 min at 37°C in the dark and were washed in medium.

PKH26-GL labeling. Cells were resuspended in 0.5 ml of diluent C (Diluent Kit for PKH26-GL, Sigma Chemical), and to this cell suspension 0.5 ml of diluent C containing 6 μl PKH26 linker was added. The cells were incubated for 2 to 5 min at room temperature in the dark. FCS (1 ml) was added to the cell suspension. After incubation for another 5 min at room temperature in the dark, the cells were washed once in PBS and twice in PBS containing 1% FCS. After labeling, cell pellets were resuspended in PBS containing 0.5% BSA and 10% heat-inactivated NHS (PBS/BSA/Δ-NHS). For conjugate formation through bispecific mAb, NALM-6 cells and K562 cells (1×10^5) were preincubated with various concentrations of W6/32*MBC1 and G250*MBC1, respectively, or a mixture of the parentals for 30 min at 4°C in the dark. Cells were washed twice with PBS/BSA/Δ-NHS. For the detection of W6/32*MBC1, opsonized NALM-6 cells were allowed to form conjugates with K562 cells for 30 min at room temperature in the dark. In the case of G250*MBC1, opsonized K562 cells were allowed to form conjugates with PIPLC-treated LS180 cells. PI in a final concentration of 1 μg/ml was added to the cell suspension to stain dead cells. After 15 min of incubation, 200 μl PBS/BSA/Δ-NHS was added. Conjugate formation was assessed on the FACScan (Becton Dickinson), using the software program FACScan. Ten thousand events were counted. $FITC^+$ cells were measured on FL1: BP530/30 nm (green fluorescence). $PKH26\text{-}GL^+$ cells were measured on FL2: BP 585/42 nm (orange fluorescence). PI^+ (dead) cells were measured on FL3: LP 650 nm (red fluorescence). Fluorescence compensation was used to correct for spectral cross-talk between the FITC and PKH26-GL signals. PI^+ (dead) cells were gated out during analysis. Discrimination between $PKH26\text{-}GL^+/PI^+$ (dead) cells and $PKH26\text{-}GL^+/PI^-$ (viable) cells was performed using the FL2/FL3 dot plot. For the detection of conjugate formation by immunofluorescent microscopy, cells were labeled with 30 μg/ml FITC or with PKH26-GL in the above-mentioned concentration.

C3 deposition

Tumor cells (2×10^5) were incubated with 100 μl mAb for 30 min at 4°C. After washing twice with PBS/BSA, the cells were incubated with 5% NHS for 10 min at 37°C. Cells were washed twice and incubated with a 1:50 dilution of FITC-labeled goat anti-human C3c for 30 min at 4°C. Subsequently, the cells were washed, and after 15 min of incubation with 1 μg/ml PI, the C3 deposition was assessed by flow cytometric analysis. The C3 deposition was calculated by subtracting the C3 deposition in the absence of mAb from the C3 deposition measured in the presence of mAb. Data are expressed in mean equivalent standard fluorescence (MESF) values. MESF values were calculated on the basis of a flow cytometry standardization kit, Quantum 25 FITC (Flow Cytometry Standards Europe, Leiden, The Netherlands).

^{51}Cr release assay

The total reaction volume of the ^{51}Cr release assay was 250 μl, and all dilutions were performed in DMEM/RPMI medium. Fifty microliters of ^{51}Cr-labeled target cells (2000 cells/well) were mixed with 100 μl mAb in round-bottom microtiter plates and incubated for 30 min at 37°C. As a homologous complement source, 100 μl of NHS was added (final concentration, 20%). As a control for complement activation, BRC (final concentration, 2.5%) was used as a heterologous complement source. Wells with NHS were incubated for 4 h and wells with BRC for 2 h at 37°C, after

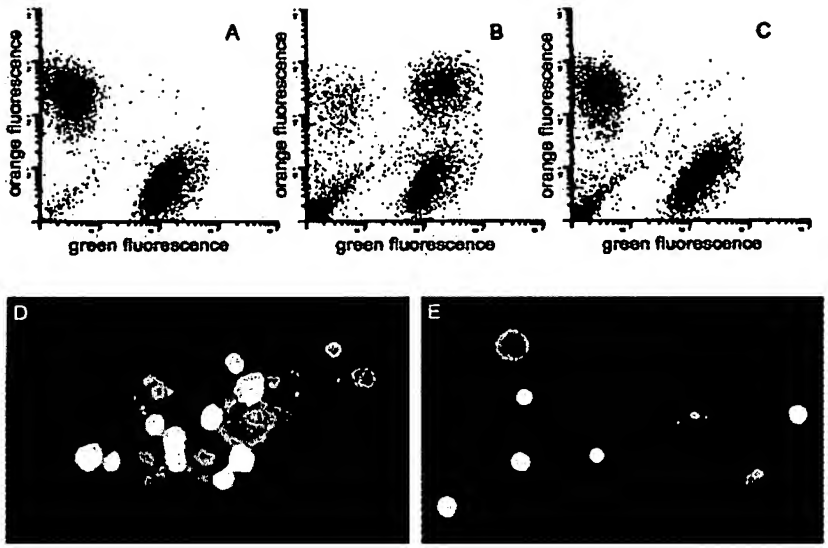

FIGURE 1. Conjugate formation through W6/32*MBC1. Flow cytometric analysis of conjugate formation. A mixture of FITC-labeled NALM-6 cells and PKH26-GL-labeled K562 cells: *A*, in the absence of mAb; *B*, in the presence of 10 μg/ml purified bi-isotypic mAb containing bispecific mAb W6/32*MBC1; and *C*, in the presence of W6/32 and MBC1 (both 10 μg/ml). Conjugate formation was confirmed by immunofluorescence microscopy: *D*, in the presence of W6/32*MBC1; and *E*, in the presence of both parental mAb. The results shown are representative of two experiments performed.

which 100 μl of supernatant was counted for ^{51}Cr release. Maximal release was defined as the release obtained by the addition of 10% (v/v) Triton X-100 to the target cells. Spontaneous release was obtained by incubating the target cells with medium. The percentage of specific release was calculated as 100 × (counts experimental release − spontaneous release)/(maximal release − spontaneous release). All tests were performed in triplicate.

Results

*Generation and selection of quadroma clones producing bispecific mAb W6/32*MBC1*

In this study, a bispecific mAb was generated, directed against both a tumor-associated Ag and CD55, and its effect on C3 deposition and tumor cell lysis in vitro was investigated. As a model, we first studied the effects of a bispecific mAb directed against CD55 and HLA class I. Quadroma clones were generated by fusion of MBC1 (anti-CD55, IgG1) and W6/32 (anti-HLA class I, IgG2a) hybridoma cells (19). Cultures were cloned in 96-well plates, each well statistically containing 0.3 cell. The supernatants of the W6/32*MBC1 quadroma clones were tested for the presence of bi-isotypic mAb by means of a bi-isotypic ELISA. Bi-isotypic mAb were produced by 90% of the quadroma clones. Fifteen clones, selected on the basis of their high production of bi-isotypic mAb, were tested for the presence of functional binding sites by flow cytometric analysis. Nine clones found to be positive for functional binding sites were selected and subcloned.

To determine the ratio between the parental and bi-isotypic mAb produced by the different quadroma subclones, the concentration of both parentals and bi-isotypic mAb was measured using isotype-specific ELISA. Quadroma clone 37A (W6/32*MBC1) was selected for further study on the basis of both production pattern and growth rate.

The presence of bispecific mAb W6/32*MBC1 in the purified bi-isotypic fraction was confirmed by its ability to form conjugates between FITC-labeled NALM-6 cells (HLA class $I^+/CD55^-$) and PKH26-GL-labeled K562 cells (HLA class $I^-/CD55^+$) (Fig. 1). Conjugate formation was assessed by flow cytometric analysis. bispecific mAb W6/32*MBC1, present in the purified bi-isotypic fraction, was able to cross-link both cell lines at mAb concentrations ranging from 1 to 10 μg/ml. At these concentrations, 28 to 39% of the labeled cell population consisted of conjugates (Fig. 1*B*). Conjugate formation was not found when the cells were incubated with a mixture of the parental mAb (Fig. 1*C*). The double negative population consisted of K562 and NALM-6 cells, which had lost their fluorochrome content as a result of the sequential washings. The results were confirmed by double-staining immunofluorescent microscopy (Fig. 1, *D* and *E*).

Bispecific mAb are able to activate the complement system

We generated bispecific mAb of which the Fc portion consisted of one IgG1 chain and one IgG2a chain. To determine whether one IgG2a chain is sufficient for activation of the complement system, a ^{51}Cr release assay was performed with NALM-6 cells, which have a high expression of HLA class I but are deficient in the expression of both CD55 and CD59. The absence of both CD55 and CD59 enabled us to use cytolysis, which is otherwise influenced by these complement regulators, as a measure for complement activation. NALM-6 cells were opsonized with bi-isotypic mAb or the parental mAb, and the specific lysis in the presence of homologous serum as a source of complement was measured (Fig. 2).

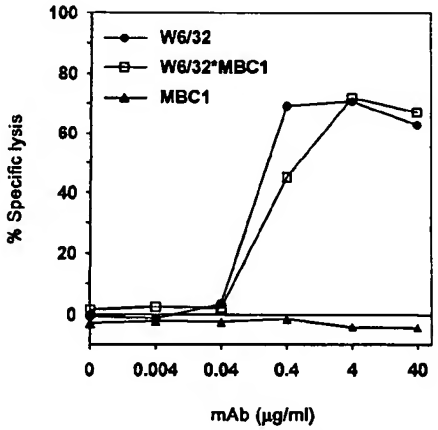

FIGURE 2. Complement activation by bispecific mAb W6/32*MBC1. ^{51}Cr-labeled NALM-6 cells were opsonized with increasing concentrations of W6/32*MBC1 or the parental mAb in the presence of NHS (5%) as a source of complement. Specific lysis was assessed as described in *Materials and Methods*. The results shown are representative of two experiments performed. The data plotted are the means of triplicate values.

Table I. *C3 deposition on renal tumor cell line SK-RC-7 in the presence of W6/32*MBC1*

mAb (μg/ml)	C3 Deposition (MESF)[a]
MBC1, 20	−21 ± 13
W6/32, 20	364 ± 90
W6/32, 20 + MBC1, 0.2	336 ± 52
W6/32, 20 + MBC1, 2	405 ± 72
W6/32, 20 + MBC1, 20	594 ± 55
W6/32, 20 + GB24, 20	325 ± 58
W6/32, 20 + BRIC229, 20	309 ± 42
W6/32, 20 + G250, 20	365 ± 57
W6/32*MBC1, 0.2	−22 ± 50
W6/32*MBC1, 2	15 ± 39
W6/32*MBC1, 20	298 ± 65
W6/32*MBC1, 200	697 ± 64*

[a] MESF, mean equivalent standard fluorescence.

* $p < 0.007$, a significant difference between C3 deposition in the presence of W6/32*MBC1 (200 μg/ml) and W6/32 (20 μg/ml), both under saturating conditions (Student's *t* test).

The parental mAb MBC1 was used as a negative control because it cannot bind to NALM-6 cells. The complement-activating mAb W6/32 induced complement activation at mAb concentrations ranging from 0.4 to 40 μg/ml, resulting in 70% lysis. Similar plateau values of specific lysis were reached at a concentration of 4 μg/ml of the purified bi-isotypic fraction of W6/32*MBC1. From these data, it was concluded that one IgG2a chain is sufficient for complement activation. Since MBC1 is able to block the function of CD55 at the site of complement activation, the effect of W6/32*MBC1 on the C3 deposition on a renal tumor cell line, both positive for HLA class I and CD55 expression, was studied.

*Enhanced C3 deposition in the presence of W6/32*MBC1*

The renal tumor cell line SK-RC-7 was opsonized with increasing concentrations of the purified bi-isotypic fraction of W6/32*MBC1 or with saturating concentrations of the two parental mAb either separately or mixed. No C3 deposition was observed in the presence of Mg-EGTA, demonstrating that complement activation was due to classical pathway activation (data not shown). The parental mAb MBC1 (IgG1) alone was not able to activate the complement system, and therefore, no C3 deposition was found (Table I). The complement-activating mAb W6/32 induced membrane deposition of C3 on tumor cells. This was further enhanced by blocking the function of CD55 with increasing concentrations of mAb MBC1. C3 deposition in the presence of G250 (IgG1) as an isotype control for MBC1 was measured, and no effect was found. This confirmed that the enhancement of the C3 deposition was due to blocking the function of CD55 through MBC1.

Replacing MBC1 with GB24, a blocking mAb for CD46, which, like CD55, regulates the complement cascade at the level of C3/C5 convertases, did not result in an increase in C3 deposition such as was seen in the presence of MBC1. This could not be due to loss of the functional blocking capacity of the mAb, as GB24 was able to block the function of CD46 on NALM-6 cells (data not shown). As expected, blocking the function of CD59, a regulator of complement activation at the level of the membrane attack complex, with BRIC229 had no effect on the C3 deposition.

Increasing concentrations of W6/32*MBC1 induced an enhancement of C3 deposition. A 92% enhancement of C3 deposition was reached in the presence of saturating concentrations of W6/32*MBC1 in comparison with C3 deposition in the presence of W6/32 alone. Furthermore, C3 deposition in the presence of saturating concentrations of W6/32*MBC1 was 17% higher than C3 deposition in the presence of saturating concentrations of a mixture of parental mAb.

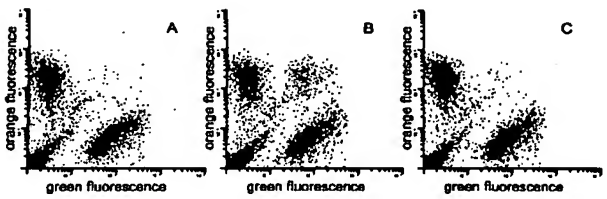

FIGURE 3. Conjugate formation through G250*MBC1. Flow cytometric analysis of conjugate formation. A mixture of PIPLC-treated, FITC-labeled LS180 cells and PKH26-GL-labeled K562 cells: *A*, in the absence of mAb; *B*, in the presence of 10 μg/ml purified bi-isotypic mAb containing bispecific mAb G250*MBC1; and *C*, in the presence of G250 and MBC1 (both 10 μg/ml). The results shown are representative of two experiments performed.

In addition, to exclude the possibility that enhanced C3 deposition was an effect of bi-isotypic mAb other than bispecific mAb W6/32*MBC1, which are present in the bi-isotypic fraction, this experiment was also performed with HPLC-purified bispecific mAb W6/32*MBC1. The results for C3 deposition in the presence of bispecific mAb were identical to the results obtained with the bi-isotypic fraction. This indicates that the bispecific mAb W6/32*MBC1, present in the purified bi-isotypic fraction, was responsible for enhanced C3 deposition. Based on these results, bispecific mAb directed against CD55 and the relatively low expressed renal tumor-associated Ag G250 were generated.

Generation of bispecific mAb directed against G250 and CD55

The selection of quadroma clones producing bispecific mAb G250*MBC1 was performed as described for the generation of W6/32*MBC1 clones. Quadroma clone V/O1.17 was selected for further study.

Glycoinositolphospholipid-anchored CD55 on $G250^+$ renal tumor cell lines cannot be removed by treatment with PIPLC (20). Therefore, LS180 cells, which express G250 when grown to confluence, were used to perform the cross-link assay with bispecific mAb G250*MBC1. After treatment with PIPLC, the expression of CD55 on LS180 cells was reduced to 5 to 10% of its original expression level.

As shown in Figure 3, bispecific mAb G250*MBC1 was able to form conjugates between PKH26-GL-labeled K562 ($G250^-$/$CD55^+$) and PIPLC-treated LS180, labeled with FITC. In the presence of 10 μg/ml bi-isotypic mAb, 11% of the labeled cell population consisted of conjugates (Fig. 3*B*). No formation of conjugates was observed in the presence of both parental mAb (Fig. 3*C*). This finding demonstrates that the interaction was due to the bispecific mAb G250*MBC1 and not to the presence of trace amounts MBC1 in the purified bi-isotypic fraction, which could have formed conjugates between the remaining CD55 on LS180 cells and CD55 on K562 cells.

*Enhanced C3 deposition in the presence of G250*MBC1 results in increased lysis*

The renal tumor-associated Ag G250 is expressed at a low density on renal tumor cell lines as compared with the expression of HLA class I. To investigate whether the approach with bispecific mAb is also effective for Ags with a low expression, bispecific mAb directed against CD55 and G250 were produced.

The effect of this bispecific mAb on C3 deposition was studied by opsonizing the human renal tumor cell line SK-RC-52 with

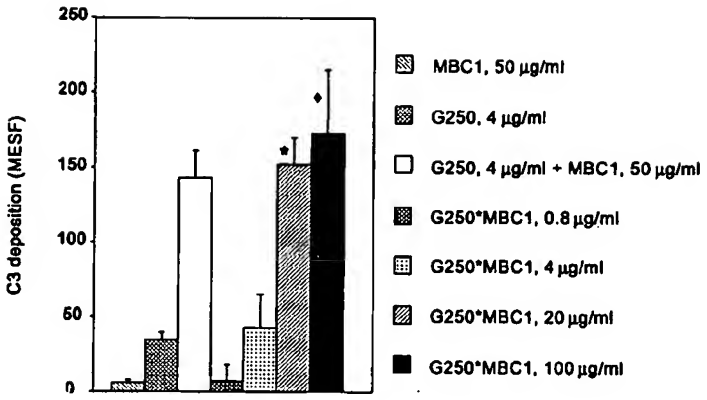

FIGURE 4. C3 deposition in the presence of G250*MBC1 or the parental mAb. SK-RC-52 cells were opsonized with increasing concentrations of G250*MBC1 or saturating concentrations of parental mAb alone or in combination. Opsonized cells were incubated with NHS (5%) as a source of complement, and the C3 deposition was assessed by flow cytometry. The results are representative of two experiments performed. Bars represent the variation between duplicate determinations *, $p < 0.03$, a significant difference between the C3 deposition in the presence of G250*MBC1 (20 µg/ml) and a saturating concentration of G250 (4 µg/ml); ♦, $p < 0.03$, a significant difference between the C3 deposition in the presence of G250*MBC1 (100 µg/ml) and G250 (4 µg/ml), both under saturating conditions (Student's *t* test).

increasing concentrations of the purified bi-isotypic fraction of G250*MBC1 or with saturating concentrations of the parental mAb either alone or mixed (Fig. 4). As expected, the parental mAb MBC1 (IgG1) alone did not induce membrane deposition of C3. The parental mAb G250 (IgG2a) alone induced a minor C3 deposition on the tumor cells, which was enhanced fourfold if CD55 was simultaneously blocked with MBC1. Increasing concentrations of G250*MBC1, ranging from 4 to 100 µg/ml, induced a corresponding 25 to 400% enhancement of C3 deposition when compared with C3 deposition in the presence of a saturating concentration of mAb G250 alone. A 21% increase in C3 deposition was observed in the presence of saturating concentrations of G250*MBC1 as compared with the C3 deposition with a mixture of both parental mAb under saturating conditions.

When this experiment was performed with HPLC-purified bispecific mAb G250*MBC1, with concentrations ranging from 0.4 to 50 µg/ml, the results for C3 deposition were identical to the results obtained with the bi-isotypic fraction. This indicates that enhanced C3 deposition was due to bispecific mAb G250*MBC1, present in the purified bi-isotypic fraction.

These observations suggest that blocking CD55 at the site of complement activation is an important factor in enhancing C3 deposition. To determine whether the enhanced C3 deposition, measured in the presence of G250*MBC1, results in an increased lysis, the human renal tumor cell line SK-RC-7 was used in a ^{51}Cr release assay. Previous investigations have demonstrated that SK-RC-7 is susceptible to complement-mediated lysis in the presence of W6/32 and homologous serum (20). G250, however, has not been able to induce lysis of SK-RC-7, due to the combination of a low expression of the tumor-associated Ag and expression of mRCA.

SK-RC-7 cells were opsonized with the purified bi-isotypic fraction of G250*MBC1 or with G250, and the ^{51}Cr release in the presence of homologous serum was measured (Fig. 5). Opsonization with W6/32 served as a control for complement activation, and plateau values of 70% lysis were reached. Neither G250 alone nor G250*MBC1 induced lysis of SK-RC-7 (Fig. 5*A*). However, if the function of CD59 was simultaneously blocked with BRIC229, G250*MBC1 was able to induce a significant twofold enhancement of lysis of SK-RC-7, when compared with G250 alone (Fig. 5*B*). The lysis of W6/32 was only slightly enhanced to 85%. Again, the results with HPLC-purified bispecific mAb G250*MBC1 were identical to those obtained with the purified bi-isotypic fraction.

The observation that the tumor cells are not lysed in the presence of G250*MBC1 alone, even though C3 deposition is higher than with G250 alone, seems to indicate that the enhanced

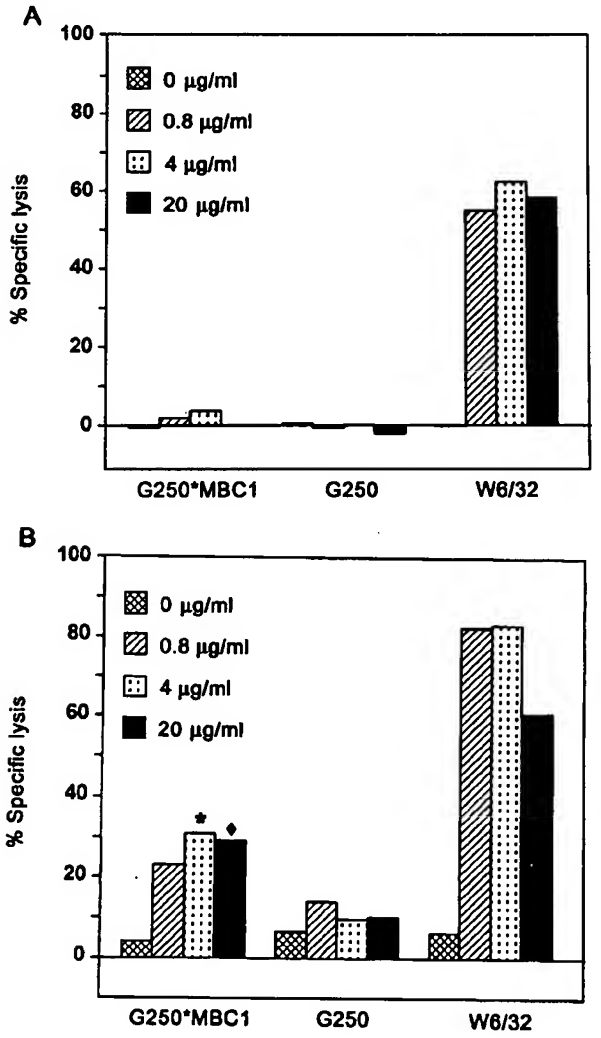

FIGURE 5. Effect of G250*MBC1 on lysis of renal tumor cells. SK-RC-7 cells were opsonized with G250*MBC1, G250, or W6/32 in the absence (*A*) or presence of 20 µg/ml anti-CD59 mAb (BRIC229) (*B*). NHS (20%) was added as a source of complement. After 4 h, the amount of ^{51}Cr in the supernatant was measured, and the specific ^{51}Cr release was measured as described in *Materials and Methods*. The results shown are representative of two experiments performed. *, $p < 0.0001$, a significant difference between lysis in the presence of G250*MBC1 (4 µg/ml) and G250 (4 µg/ml); ♦, $p < 0.008$, a significant difference between lysis in the presence of G250*MBC1 (20 µg/ml) and G250 (20 µg/ml) (Student's *t* test).

membrane deposition of C3 induced by G250*MBC1 is not sufficient to overcome the action of CD59. However, when CD59 is functionally blocked simultaneously, the enhanced C3 deposition induced by G250*MBC1 is accompanied by an increase in lysis compared with the parental mAb G250. The above results show that the bispecific mAb directed against G250 and CD55 is more efficient in inducing membrane deposition of C3 and lysis of tumor cells than mAb G250 alone.

Discussion

Immunotherapeutic treatment of RCC includes therapy with biologic response modifiers, adoptive cellular therapy (1, 4, 21), and the administration of complement-activating Abs (5). A possible explanation for the disappointing response rate in patients with RCC so far may be a lack of inflammatory response at the tumor site, which could be due to the expression of mRCA by renal tumor cells (20, 22).

In the present study, a bispecific mAb simultaneously directed against a tumor-associated Ag and CD55 was generated, and its effect on C3 deposition and lysis were investigated on human renal tumor cell lines. In a rat model, it has been shown that blocking the 5I2 Ag (a rat counterpart of mouse Crry/p65; a functional counterpart of human CD46 and/or CD55) resulted in changes in blood pressure, deposition of C3 in the vasculature, and an increase in vascular permeability. These effects are thought to be due to the generation of anaphylatoxins C3a and C5a (23); they were not seen after blocking the function of the rat counterpart of CD59 with mAb 6D1. A further study, using a rat kidney perfusion model, revealed renal injury and leukocyte infiltration when the function of 5I2 Ag was inhibited, but not when rat CD59 was blocked (24). In our study, we chose CD55 as a target because blocking CD55 in vivo seems to be more effective than blocking of CD59.

Since HLA class I is highly expressed on several human renal tumor cell lines, we first studied the applicability of our approach with a bispecific mAb directed against HLA class I and CD55. Although the Fc portion of this mAb (W6/32*MBC1) contained only one IgG2a chain, this proved sufficient to induce complement activation. Subsequently, W6/32*MBC1 and the parental mAb alone or mixed were compared with respect to C3 deposition on the human renal tumor cell line SK-RC-7. The highest C3 deposition was observed in the presence of W6/32*MBC1. We observed that replacement of MBC1 by GB24, a blocking mAb for CD46, had no effect on the C3 deposition. This finding seems to confirm prior data that CD46 is mainly involved in the regulation of the alternative pathway of complement activation (25, 26).

Based on the results with W6/32*MBC1, we generated a bispecific mAb directed against CD55 and the relatively low expressed renal tumor-associated Ag G250. The expression of the G250 Ag on SK-RC-52 is ~3.5-fold lower than that of HLA class I on SK-RC-7 (20). Only a minor C3 deposition was measured in the presence of mAb G250 alone, which is most probably due to the low antigenic density of the G250 Ag and the presence of mRCA on renal tumor cells. A significant enhancement of the C3 deposition was measured in the presence of G250*MBC1, suggesting that blocking CD55 at the site of complement activation is essential for efficient deposition of C3. The observation that bispecific mAb are able to induce a higher C3 deposition than a mixture of the two parental mAb under saturating conditions might be explained by the proximity of the Fab portion directed against CD55 to the Fab portion directed against the tumor-associated Ag. In this way, the CD55 molecule closest to the site of complement activation is blocked.

In previous experiments, G250, in contrast to W6/32, has not been able to induce complement-mediated lysis of SK-RC-7 cells, probably due to low C3 deposition. Despite an enhancement of the C3 deposition in the presence of G250*MBC1, no lysis of SK-RC-7 cells was observed. Lysis was seen only if CD59 was blocked, suggesting that the enhancement of the C3 deposition was not sufficient to overcome the inhibitory actions of CD59. When CD59 was blocked, the enhanced C3 deposition in the presence of G250*MBC1 resulted in an increased lysis compared with lysis in the presence of G250.

In vivo, however, direct lysis of tumor cells by formation of the membrane attack complex may not be the most important contribution of complement to destruction of tumors. Enhanced C3 deposition on the surface of tumor cells seems to be of greater importance in vivo, as it provides a stimulus for phagocytosis and killing by effector cells through Ab-dependent cellular cytotoxicity (27).

An important problem in the use of blocking Abs directed against mRCA is how to target tumor cells specifically and avoid the indiscriminate attack of bystander cells by complement. Junnikkala et al. (28) used an avidin-biotin system and showed that it is possible to target complement attack specifically to human melanoma cells in heterogenous cell mixtures without causing significant damage to bystander cells. To target tumor cells specifically, bispecific mAb must be generated with a far higher affinity for the tumor-associated Ag than for the complement regulator, as CD55 is also expressed on normal cells (29).

It has been observed that mRCA are not always uniformly expressed (22, 30). With regard to RCC, we have previously shown using flow cytometry that all renal tumor cell lines tested expressed CD46, CD55, and CD59 (20). Using immunohistochemistry, we observed that these mRCA are expressed on RCC (data not shown). For immunotherapy with bispecific mAb, the expression of a tumor-associated Ag is essential because it is required to target the bispecific mAb to the tumor site. Heterogeneity of CD55 expression probably will not influence the efficiency of bispecific mAb, because we have shown that bispecific mAb activate the complement system to a similar level as the complement activating parental Ab. Therefore, we believe that the bispecific mAb G250*MBC1 is equally effective as mAb G250 alone on $G250^+/CD55^-$ tumor cells and more effective in C3 deposition than mAb G250 on $G250^+/CD55^+$ tumor cells. In addition, it is known that proximal tubular epithelial cells are able to produce components of the complement system (31–33). Therefore, we deduce that renal tumors, which are derived from these epithelial cells, may be particularly susceptible to treatment with bispecific mAb, because RCC express CD55 and complement components are available locally. Patients suffering from other malignancies may also benefit from treatment with bispecific mAb, which block mRCA. Juhl et al. (34) have shown that expression of mRCA on gastrointestinal tumor cells inhibits complement dependent cytotoxicity and release of C3a, thereby limiting the therapeutic potential of mAb 17-1A. This finding supports our concept that a bispecific Ab directed against a tumor-associated Ag and CD55 may improve immunotherapeutic treatment of malignancies.

In conclusion, we have been able to generate a bispecific mAb that is simultaneously directed against the renal tumor-associated Ag G250 and the mRCA CD55. This bispecific mAb enhances C3 deposition on tumor cells in comparison to C3 deposition seen in the presence of the parental mAb G250 alone. The increase in C3 deposition also resulted in an increase in tumor cell lysis, although only if CD59 was blocked simultaneously. We have shown, in vitro, that bispecific mAb G250*MBC1 is more effective than mAb G250, although whether this also holds true for the in vivo

situation needs to be assessed. We propose that bispecific mAb G250*MBC1 deserves to be considered as the next step in improving the efficacy of immunotherapy of RCC.

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